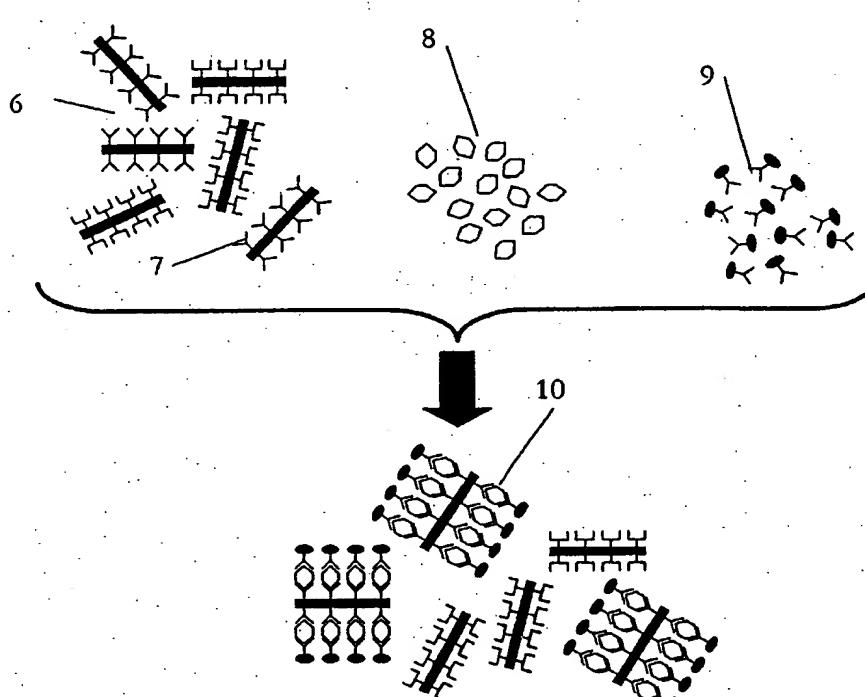


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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ :	A2	(11) International Publication Number:	WO 00/16893
B01J 19/00		(43) International Publication Date:	30 March 2000 (30.03.00)
(21) International Application Number: PCT/GB99/03109 (22) International Filing Date: 17 September 1999 (17.09.99) (39) Priority Data: 9820163.5 17 September 1998 (17.09.98) GB		(81) Designated States: AU, CN, IN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
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(54) Title: BIO-ASSAY TECHNIQUE			
(57) Abstract			
<p>The present invention relates to a system for carrying out parallel bioassays. Micro-fabricated labels (7) are made to each carry a biochemical test, many different labels are mixed together with an analyte sample (8). A device that reads the individual labels isolates the results of the individual tests. The microfabricated labels have a surface layer of anodised metal and are produced by anodising, lithographic patterning and etching steps. Aluminum is the preferred metal.</p>			
			

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Bio-assay Technique

Field of the Invention

This invention relates to the field of micromachined or microfabricated coded substrates, particularly but not exclusively for use as a parallel bioassay technique.

Background

Massively parallel bioassay tests are the enabling techniques that have made the majority of recent advances in genetics, screening and drug discovery possible. Thousands or millions of tests that previously were carried out one by one may now be combined into a single experiment yielding thousands or millions of results. The key to this process has been the development of techniques that allow the results of the many different tests to be separated from each other.

15 A number of existing techniques are described below. The techniques consist of labelling each of the constituent experiments in a manner that can be read after the experiment has completed. Labels used at present include the position of the experiment on the surface of a test chip and the fluorescent spectrum of a particle to which the experiment is bound.

20 Affymetrix's GeneChip probe array is a DNA sequence testing chip, where tens of thousands of different DNA probes are located at known points on a large 2D array. The fabrication process is described in, for example US 5,744,305 or US 5,143,854. Standard DNA hybridisation techniques are used in a chamber above the chip, and the test results are read out optically by the positions of fluorescent dots on the array (see, for example, US 25 5,578,832). The combinations of tests are pre-determined during the manufacture of the chip.

Luminex Corporation's FlowMetrix system makes use of coded microspheres, 6.5 µm in diameter. Each bead incorporates red and orange fluorophores to make up the code. Eight different intensities are possible, allowing 64 different bead types. A green fluorescent marker is used for the probes. This system is described in US 5,736,330. The system has a relatively small number of codes, and requires complex, multi-wavelength optics on flow cytometers to read the codes and fluorescence of the test.

NanoGen has a semiconductor-based microchip array, APEX, which is aimed specifically at DNA binding and sequencing experiments. NanoGen's chip is programmable by the end user with different arrays of DNA probes (see, for example, US 5,605,662 and US 5,929,208).

There are a number of other particle or substrate-based assay techniques under the general heading of Combinatorial Chemistry. For examples, WO 96/24061 describes a library of tests using radio-frequency identification tags. WO 97/32892 describes a composite support for use a combinatorial chemistry substrate. GB 2306,484 describes two-part support particles for combinatorial chemistry.

Summary of the Invention

This invention describes a system for carrying out massively parallel multiple bioassay tests in a low-cost, fast and convenient manner. The scheme involves making a suspension (an assay) containing many thousands of different types of micro-machined coded labels, (micro-labels), each code carrying a different biochemical test or probe.

An assay is constructed by mixing together suspensions of chosen sets of active micro-labels. Assays are customised to particular applications, independently of the original fabrication of the micro-labels.

The sample under test is marked with a fluorescent label and incubated with the assay. Only micro-labels with probes that bind to the fluorescent sample molecules will become fluorescent.

The micro-labels are fabricated from an anodisable material such as aluminium, initially deposited onto a planar substrate with a soluble release layer. The metal surface is anodised before patterning. This allows the attachment of a wide range of biochemically active agents for use as highly selective probes.

Standard optical lithography and dry etching is used to pattern the aluminium into separate micro-labels. The code is stored on the micro-labels as a series of holes using coding

schemes similar to those found on bar codes. The biochemical probes may be attached to the surface either before or after the lithography step. The micro-labels are then released into an aqueous suspension. Each different micro-label code has a unique probe associated with it. Micro-labels with up to 100,000 different variants have been demonstrated.

A flow-based reader system, similar to a flow cytometer, draws through thousands of micro-labels per second, reading both the bar code and the result of the test. The test result is measured by the degree of fluorescence. An alternative planar reading system, in which the micro-labels are plated out onto a flat substrate, uses fluorescence microscopy and image processing to read the results of the tests.

Brief Description of the Drawings

Embodiments of the invention will now be described, by way of example, with reference to the accompanying drawings, in which:

Figure 1 illustrates a single micro-label incorporating a transmission optical barcode,
Figure 2 illustrates a parallel bio-assay comprising two binding event experiments,
Figure 3 illustrates a wafer scale fabrication process for micro-labels,
Figure 4 illustrates a porous structure resulting from anodic oxidisation of an aluminium surface,
Figure 5 illustrates a flow through micro-label reading device,
Figure 6 illustrates the use of orthogonal interrogation beams to enable labels to be read regardless of rotation

Detailed Description

Micro-labels

Figure 1 shows a micro-label, 1, in the form of a micro-machined miniature optical bar code, made from aluminium. The bar code is formed by a series of holes, 2, in the aluminium.

Each micro-label of this type is about 100 μm long, 3, by 10 μm wide, 4, by 1 μm thick, 5, and is capable of storing up to 100,000 different codes. Around 10 million such micro-

labels can be fabricated on a single 6-inch diameter substrate. Different lengths of micro-label, from 40-100 μm , carrying between 2 and 5 decimal digits of data, have been fabricated. Each different code is associated with a unique biochemical probe. Coding schemes such as those used in EAN and UPC bar codes are used, to provide strong error checking when the codes are read.

Probes and Assays

With reference to Figure 2, an assay, 6, of micro-labels, is constructed by mixing together suspensions of chosen sets of active micro-labels. Each different code has a unique biochemical probe associated with it, which binds to a specific type of molecule. Binding reactions may be selected from the group consisting of antibody-antigen, enzyme-substrate, enzyme-receptor, toxin-receptor, protein-protein and avidin-biotin.

The sample under test, 8, is marked with a fluorescent label and incubated with the assay. Only micro-labels, 7, with probes that bind to the fluorescent sample molecules will become fluorescent, 10. The result of the test is measured by the degree of fluorescence of different types of micro-labels.

An example of an application of the present invention is the screening of serum for a selection of specific antibodies in a parallel bioassay. Antigens to the antibodies are used as probes and bound to micro-labels marked to identify these antigens. The micro-labels are incubated with the sample and then incubated with an antibody specific fluorescent label. The micro-labels are then passed through a reader that measures the degree of fluorescence of the labels and their identity. Correlation of the identities of the micro-labels with their degree of fluorescence provides indication of the binding of antibodies in the sample with the surface bound antigens.

Micro-label Fabrication

The fabrication process for the micro-labels will now be described with reference to Figure 3.

A substrate material, 11, such as a silicon wafer, is first coated with a soluble release layer, 12. In the preferred embodiment, this is a spin-coated layer of polymethyl methacrylate resist, baked at 150°C to drive off the solvents.

5 A layer of aluminium, 13, 1 µm thick, is deposited onto the substrate. This is achieved using a standard vacuum sputter coating technique, commonly used in semiconductor device fabrication.

10 The aluminium layer, 13, is anodised, 14, at a voltage of 30V in 4% phosphoric acid, using a pure aluminium cathode, for 30s, at a current density of 10 mA/cm². This leads to a 100 nm anodised layer, 15, with a pore size of around 40 nm.

15 At this stage (depending on the application) the aluminium surface may be coated with probe molecules, 16, by immersing the surface in an aqueous solution/suspension of the molecules.

20 The substrate is spin-coated with conventional optical resist, 17, Shipley S1813. The label pattern, 18, is exposed using a hard-contact optical mask, and developed using aqueous alkaline developer (MF319). The pattern is transferred into the aluminium layer using reactive ion etching with SiCl₄.

If the probe attachment is to be carried out after the lithography, the optical resist, 17, may be removed at this stage using a solvent such as isopropyl alcohol. This leaves the release layer, 13, intact, with the patterned aluminium layer, 20, on top. The probe molecules, 16, 25 may then be attached as described above, whilst the micro-labels are still attached to the substrate.

30 The micro-labels with attached probes are released, 21, from the substrate using a solvent such as acetone. Dilution and filtration leaves the micro-labels, 22, in an aqueous suspension ready for combination into an assay.

The fabrication process is suitable for a wide variety of shapes of micro-labels with holes in, including both the substantially linear type of Figure 1 and other forms of square, rectangular and round micro-labels.

5 **Anodisation and Surface Chemistry**

Proteins bind only weakly with an untreated aluminium surface when incubated in an aqueous solution. By modifying the surface this binding can be selectively enhanced to control when the binding occurs. This is important because it allows the probe molecules to be bound strongly to the surface at time of manufacture whilst maintaining weak non-specific binding of the fluorescent target molecules during the test. In this way the discrimination of the test is maximised.

10 There exists a very wide body of knowledge, gained in the first half of this century, on surface protection of aluminium components through the use of electrochemical processes.

15 Methods for growing porous surfaces are well known, as are processes for sealing these surfaces. We have exploited this knowledge to develop a relatively simple process that grows an adsorbing surface with well controlled porosity and depth. This surface binds the chosen proteins well soon after treatment, but heals with time to prevent further binding.

20 In order to generate the appropriate anodised surface morphology, it is necessary to understand the structure of typical of biomolecules used as probes. Cryogenic atomic-force-microscopy allows direct imaging of such biomolecules. For example, Immunoglobulin-G (IgG) has a Y-shaped structure. The size of the IgG molecule is approximately 40nm across, which can be considered to be a typical size.

25 With reference to Figure 4, during anodisation, an aluminium oxide layer, 23, grows on the surface of the aluminium. This oxide layer grows in a two dimensional hexagonal cell structure, 24, the size of each cell depending on the electrochemical forming voltage. In the centre of each cell, a pore, 25, forms with an aperture smaller than the width of the cell, 26. As the anodisation process progresses, the thickness, 27, of the oxide layer and the depth of the pores, 28, increase, but the cell width, 26, and the pore aperture, 28, remains constant.

Reading Systems

Two classes of reading system have been developed. These are based on flow cells, and on planar imaging of plated-out micro-labels.

5 The flow-cell reader, shown schematically in Figure 5, utilises a design that induces the micro-labels, 1, to flow down the centre of a tube, 29 through a defined reading zone, 30. Normally, elongated particles in flow would have a tendency to tumble. However, by using an accelerating sheath fluid, 31, and injecting the micro-labels into the centre of this flow, 32; a hydrodynamic focussing effect is achieved that causes all the micro-labels to align, and pass through a well-defined focal point, 30, somewhere downstream of the injection point, 32.

At the hydrodynamic focal point, 30, two orthogonal focused beams, 33,34, of laser light from a 488 nm argon-ion laser interrogate the micro-labels. This is detailed in Figure 6.
15 The use of two orthogonal interrogation beams enables the interrogation of micro-labels, regardless of their rotation with respect to the frame of reference of the flow tube. The label, 1, in Figure 6, is shown at an angle of 45° to both incident beams, which is the worst possible case. The geometry of the micro-labels, shown in Figure 1, means that light is still transmitted through the holes in the label, 2. The holes modulate the transmitted intensity
20 at the detector, 35, as the label passes through, generating a serial stream of information that is analysed in the same way as a conventional bar code. Simultaneously, the degree of fluorescence is measured, using an epi-fluorescence detector, 36. This is correlated with the code on the label. High numerical-aperture optics (microscope objective lenses) are used to achieve the desired resolution. The optimal flow-cell design has flat walls, to avoid the use
25 of custom cylindrical imaging elements.

Once sufficient micro-labels have been read the reader calculates the results of all the tests. This number required is typically between 10 and 100 copies of each type of micro-label, to enable statistical analysis to be used. The test results show the mean and standard deviation
30 of the fluorescence for each type of probe.

In a planar reading system, the micro-labels are plated out onto a flat substrate. This is either a filter substrate, or a transparent substrate. Conventional fluorescence microscopy

is used to analyse the plated substrate systematically. An image-processing system captures pictures of each field of the substrate. Transmitted light is analysed separately from fluorescent light. Each micro-label is identified from its transmitted light profile, and the fluorescence signal integrated over the surface of the label is recorded. Once again, between 10 and 100 of each type of micro-label are required to give a good statistical analysis.

Applications

A typical application uses a suspension of probes for a number of different antibodies (e.g. hepatitis, HIV). A reading system takes a drop of blood from a patient, labels it with fluorescent marker, and incubates it with the probe suspension for a few minutes. The suspension is fed through a microfluidic detector system.

Further Embodiments

Micro-label designs for planar reading systems are not limited to the linear designs described above, which are primarily intended for flow-through reading systems. Planar systems can use 2D patterns, fabricated on labels which are closer to being square or rectangular, rather than linear. Coding schemes similar to conventional 2D barcodes are then used. The outline of the label also gives information, particularly about orientation.

Labels incorporating magnetic material can use magnetic separation. This is useful if the sample being tested contains solid matter of similar size to the labels that could contaminate the results.

Micro-labels can be fabricated using any substrate that can be coated with an anodisable metal such as aluminium. This is particularly attractive when a mass-production method such as embossing of an aluminium-coated plastic is used to fabricate the micro-labels.

Claims

1. A solid support for biochemical (binding experiment) assays with an anodised metal surface layer
2. A support according to claim 1 wherein the metal surface layer is aluminium
3. A support according to claim 1, with a modification to enhance the affinity of the reagents of said binding experiments
4. A method of fabricating supports according to claim 1, comprising sputter coating of a flat surface with the metal comprising the support, lithographic patterning of the metal, and etching of the metal to leave individual supports
5. A method according to claim 4, whereby the said surface consists of a layer of soluble material on a rigid support, thereby allowing the supports to be released by solvation of the soluble material
6. A method according to claim 5 whereby the soluble material is a resist
7. A support according to claim 2, whereby the modification results in a porous surface
8. A support according to claim 7, whereby the pore size is approximately matched to the size of the biochemically active molecules to be bound
9. A method of fabricating the support according to claim 3, wherein said modification is by electrochemical means
10. A method according to claim 9, wherein said modification is by anodic oxidation of the support surface
11. A method according to claim 10, wherein said anodisation is carried out at a voltage of up to 150 V

12. A method according to claim 11 whereby said anodisation is carried out at a voltage from 4 V to 30 V
13. A support according to claim 1, wherein said support is preferentially smaller than 100 µm in length by 100 µm in width by 100 µm in thickness
14. A support according to claim 1, incorporating a spatially varying pattern for identification purposes
15. An optical reader for reading the patterns and identifying the supports according to claim 14
16. Supports according to claim 14, wherein said pattern is a barcode
17. Supports according to claim 16, wherein said barcode is a linear barcode
18. A reader according to claim 15 operating by means of transmission optics
19. A reader according to claim 18 wherein said supports are transported through an optical read volume by a fluidic system
20. A process whereby the results of many binding experiments are separated from one another according to the identities of substrates according to claim 14 to which one member of said binding pair is attached, as determined by a reader according to claim 15.
21. A process according to claim 20 wherein said binding pairs are selected from the group consisting of antibody-antigen, enzyme-substrate, enzyme-receptor, toxin-receptor, protein-protein and avidin-biotin
22. A process according to claim 20, wherein the attached member of said binding pair is fluorescently labelled

23. A support according to claim 14 in which the pattern comprises a series of holes in the support
24. A reader according to claim 18 in which there are two substantially orthogonal light transmission paths
25. A reader according to claim 24 incorporating one or more fluorescence detectors

FIGURES

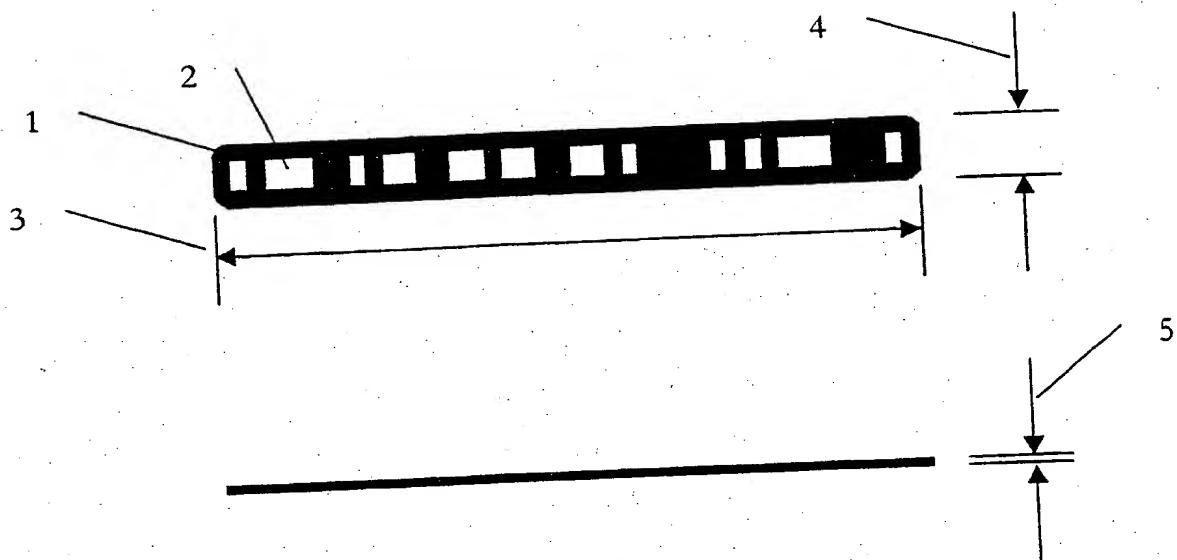


Figure 1

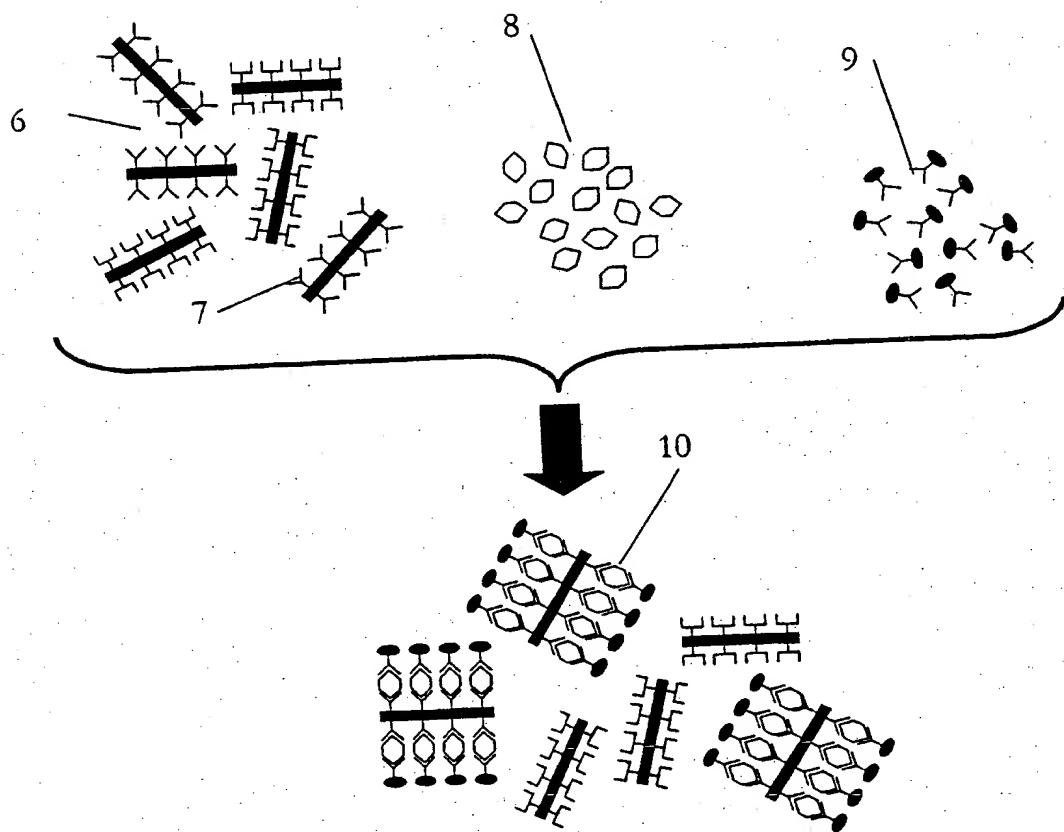


Figure 2

2/4

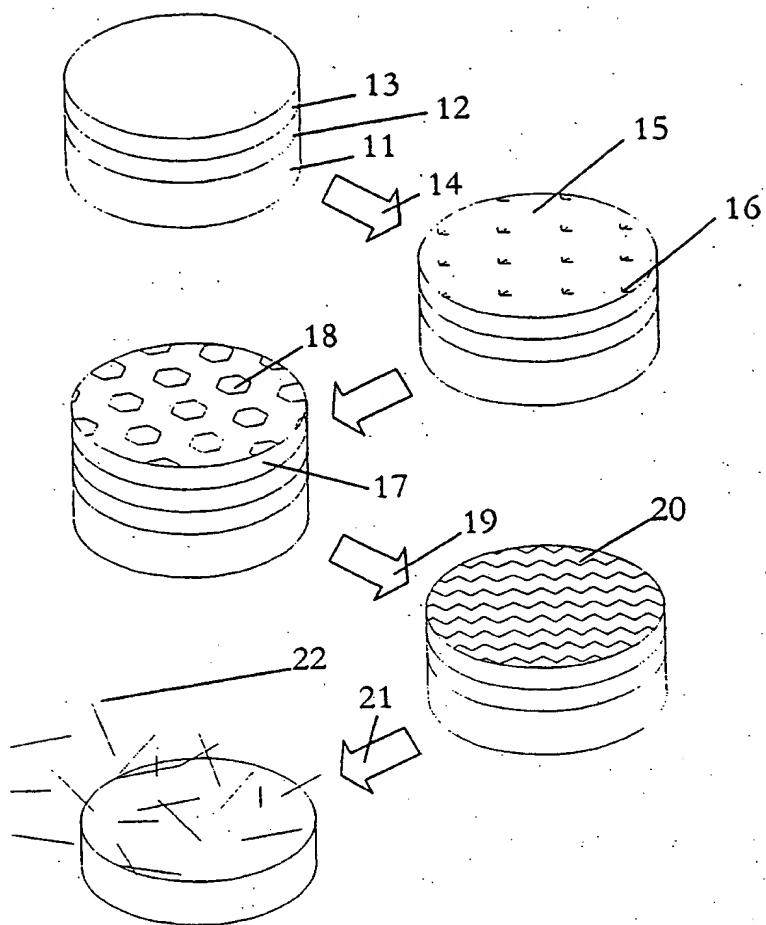


Figure 3

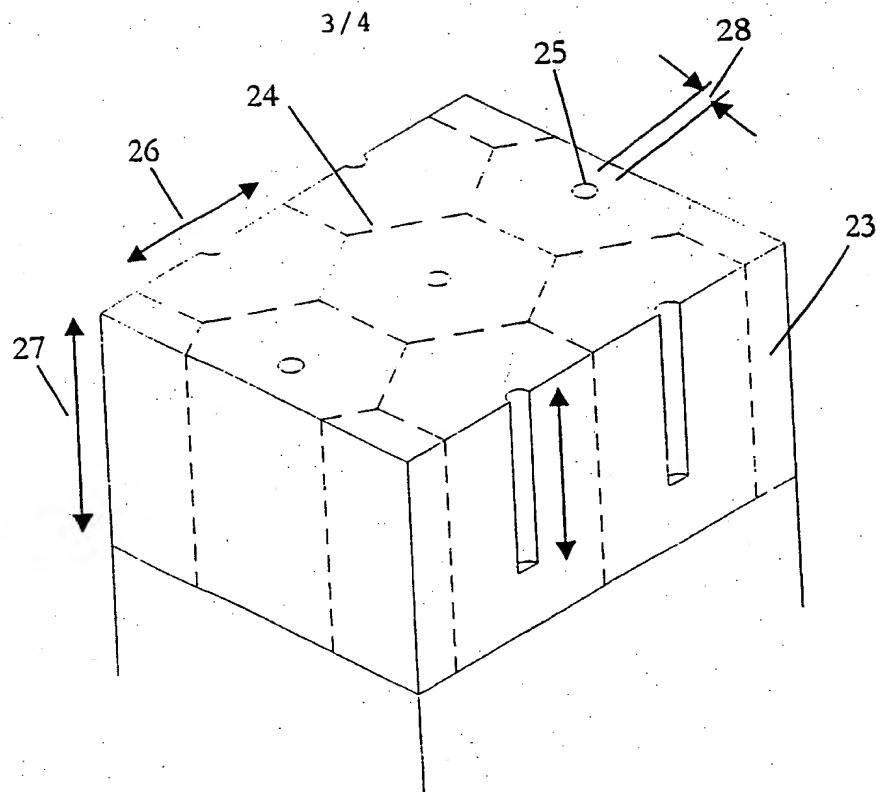


Figure 4

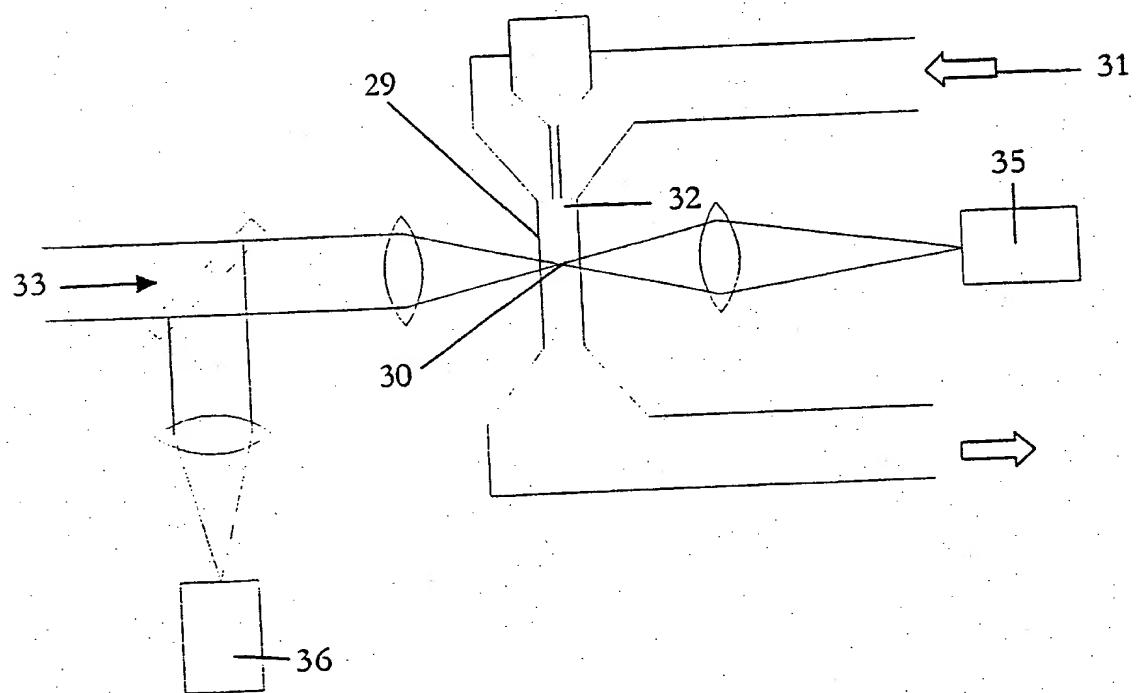


Figure 5

4 / 4

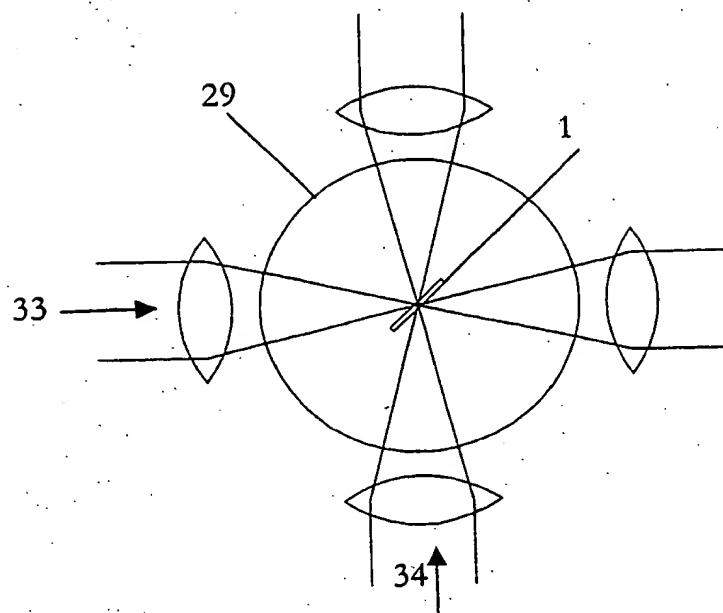


Figure 6

INTERNATIONAL SEARCH REPORT

Inte Jonal Application No
PCT/GB 99/03109

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J19/00 G06K19/06 G01N33/53 C25D11/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 B01J G06K G01N C25D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 395 300 A (ALCAN INT LTD) 31 October 1990 (1990-10-31) abstract	1-3,7-13
A	page 4, line 22 - line 30; claims 1,10,12,17-26; figures 3-5; examples 1,3 ---	20-22
X	US 5 129 974 A (AURENIUS CARL A D) 14 July 1992 (1992-07-14) abstract	1-3,7,8, 13,14
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A	page 6, paragraph 3 -page 7, paragraph 1; figure 1 ---	4
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RIGBY W R ET AL.: "An anodizing process for the production of inorganic microfiltration membranes" TRANSACTIONS OF THE INSTITUTE OF METAL FINISHING, vol. 68, August 1990 (1990-08), pages 95-98, XP002130485 the whole document	1-3,7-14
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Y	page 19, line 24 -page 21, line 2; figure 4	25
A	the whole document	13,14, 20-23
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Y		25
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A	LAURELL T ET AL: "ENHANCED ENZYME ACTIVITY IN SILICON INTEGRATED ENZYME REACTORS UTILIZING POROUS SILICON AS THE COUPLING MATRIX" SENSORS AND ACTUATORS B,CH,ELSEVIER SEQUOIA S.A., LAUSANNE, vol. 31, no. 3, 1 March 1996 (1996-03-01), pages 161-165, XP000630913 ISSN: 0925-4005 the whole document	1-3,7-14
		-/-

INTERNATIONAL SEARCH REPORT

Int'l. Jpnal Application No
PCT/GB 99/03109

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 24061 A (ONTOGEN CORP) 8 August 1996 (1996-08-08) cited in the application page 47, line 6 -page 49, line 29; claims 14-53; figure 7	14,20-23

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-14,16,17,20-23

Claims 1-3,7,8,13,14,16,17,23, related to a solid support with an anodised metal surface layer,
Claims 4-6, related to a method of fabricating such support,
Claims 9-12, related to a method of fabricating such support,
Claims 20-22, related to a process of separating results of many binding experiments.

2. Claims: 15,18,19,24,25

Claims 15,18,19,24,25, related to an optical reader.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/03109

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